Differential permissivity of human cerebrovascular endothelial cells to enterovirus infection and specificities of serotype EV-A71 in crossing an in vitro model of the human blood–brain barrier

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Human cerebral microvascular endothelial cells (hCMEC/D3 cell line) form a steady polarized barrier when cultured in vitro on a permeable membrane. Their susceptibility to enterovirus (EV) strains was analysed to investigate how these viruses may cross the blood–brain barrier. A sample of 88 virus strains was selected on phylogenetic features amongst 43 epidemiologically relevant types of the four EV species A–D. The EV-A71 genome was replicated at substantial rates, whilst the infectious virus was released at extremely low but sustained rates at both barrier sides for at least 4 days. EV-A71 antigens were detected in a limited number of cells. The properties of the endothelial barrier (structure and permeability) remained intact throughout infection. The chronic EV-A71 infection was in sharp contrast to the productive infection of cytolytic EVs (e.g. echoviruses E-6 and E-30). The hCMEC/D3 barriers infected with the latter EVs exhibited elevated proportions of apoptotic and necrotic cells, which resulted in major injuries to the endothelial barriers with a dramatic increase of paracellular permeability and virus crossing to the abluminal side. The following intracellular rearrangements were also seen: early destruction of the actin cytoskeleton, remodelling of intracellular membranes and reorganization of the mitochondrion network in a small cluster near the perinuclear space.

INTRODUCTION

Members of the genus Enterovirus (EV), family Picornaviridae, form a large group of non-enveloped enteric viruses, of which >100 different serotypes are human pathogens classified within four taxonomic species (EV-A to -D). Human EVs are transmitted through faecal/oral and respiratory routes, and they actively replicate in the mucosa of the throat and intestinal tract. Viral invasion of the intravascular space or viraemia may result in spreading to sites such as the skin, heart and central nervous system (CNS).

The most common clinical manifestation associated with CNS EV infections is aseptic meningitis. Encephalitis, cerebellitis, myelitis and poliomyelitis are also observed, but less frequently (Khetsuriani et al., 2006; Antona et al., 2007). There is evidence for haematogenous and neural routes of poliovirus (PV; EV-C) dissemination, and both involve viraemia (Sabin, 1956); the two routes are not mutually exclusive. It was suggested that the virus is conveyed through the neural pathway by retrograde axonal transport from infected tissues to the CNS via peripheral nerves (Ren & Racaniello, 1992; Gromeier & Wimmer, 1998). In mouse models, PV can be transported along nerves through either a process involving the CD155 receptor or in a receptor-independent manner (Ohka et al., 2012). Investigations with different animal models have revealed a possible link between neurological injury caused
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(a) Test of H0 (n=88 in duplicate)
Spearman’s ρ 77%
P<0.001

(b) EV genome copy number per cell in 24 h (log10)

(c) EV infectious virus per cell in 24 h (log10)

(d) Cumulative 24 h cell deaths (%)

Cell fragments
Necrotic cells
Apoptotic cells
Fig. 1. Heterogeneity of EV infection in hCMEC/D3 cells. Replication in hCMEC/D3 cells of EV strains selected amongst species, types and genogroups was examined at 24 h p.i. (a) Data represent the mean of two independent assays, and given as the number of viral genome copies per cell (x-axis) and of infectious particles per cell (y-axis). Correlation between the yield of virus genome and yield of infectious virus is indicated. (b) Replication kinetics of virus strains of four EV types in hCMEC/D3 cells. Data represent the mean of three independent replicates for each virus: E-30 (●), E-6 (▲), E-12 (●) and EV-A71 (○). (c) Susceptibility spectrum of the hCMEC/D3 cell line to EVs estimated as the proportion of infected cells at 6 h.p.i. A sample of 24 viral strains representing 19 different types was tested. Green and blue fluorescence indicate VP1 protein and nuclei, respectively. Bar, 100 μm. Data represent the mean ± SD of three experiments. (d) Comparison of cell mortality rates at 24 h.p.i. (n=15 EV strains). Data represent the mean ± SD of four independent experiments. The dashed blue line indicates the cell mortality rate (10%) in mock-infected cells (NoV).

by EV-A71 and retrograde axonal transport of the virus to the CNS (Chen et al., 2007; Khong et al., 2012). The occurrence of encephalomyelitis and subsequent paralysis associated with these two EVs could be explained by transport via the neural pathway, but the inefficiency of the axonal transport limits virus access to the CNS (Lancaster & Pfeiffer, 2010).

Alternatively, a virus in the bloodstream may enter the CNS by crossing the vascular endothelium in the meninges, the choroid plexus or the brain parenchyma. PV-1 was suggested to cross the mouse blood–brain barrier (BBB) independently of the CD155 receptor and of infected leukocytes (Yang et al., 1997). During EV-A71 infection, viraemia early after the onset of disease was related to severe CNS involvement in young children (Cheng et al., 2014) and to neurological impairment in experimentally infected rhesus monkeys (Zhang et al., 2011). In neonates infected with coxsackievirus B3 (CV-B3; EV-B), a high blood viral load was related to greater disease severity (Yen et al., 2007). Using sensitive quantitative gene amplification techniques to amplify viral RNA from the cerebrospinal fluid (CSF), it was possible to detect evidence of EV infection of the CNS in patients with aseptic meningitis early after the onset of disease in both children and adults (Volle et al., 2014). Our current knowledge about the processes involved in EV immigration into the CSF is still limited. As this inflammatory disease of the subarachnoid space is common to most EV serotypes, it is assumed that viruses travel through the blood, breaching the blood–CSF barrier either directly or through infected leukocytes. A number of studies showed that PV, CV-B3 and EV-A71 could infect various immune cells (Eberle et al., 1995; Vuorinen et al., 1996; Haddad et al., 2004; Wahid et al., 2005a, b; Tabor-Godwin et al., 2010). These data suggest a role of infected leukocytes in EV dissemination to the CNS through a ‘Trojan horse’ process. In vitro studies showed the susceptibility to different EVs of human vascular endothelial cells of different tissue origins (Conaldiri et al., 1997; Saijets et al., 2003; Zanone et al., 2003; Liang et al., 2004; Bozym et al., 2010; Ylipaasto et al., 2010). In addition, PV-1 and CV-B3 induced different cell signalling and endocytosis pathways in human brain microvascular endothelial cells, which was suggestive of possible variations in BBB crossing between EV types (Coyne et al., 2007; Bozym et al., 2010).

In this study, we used the hCMEC/D3 human cerebral microvessel endothelial cell line as a model of brain endothelium (Weksler et al., 2005, 2013). The hCMEC/D3 cells were used as a model for investigating whether or not EVs could breach an endothelial barrier. We first examined the susceptibility of hCMEC/D3 cells to infection by a set of 43 EV serotypes and then analysed the ability of a subset of EVs to cross endothelial barriers.

**RESULTS**

**Susceptibility of hCMEC/D3 cells to 43 EV types**

We used a first set of 88 virus strains (Table S1, available in the online Supplementary Material) chosen within EV-B (n=37 types), EV-A (n=5), EV-C (n=1) and EV-D (n=1). Susceptibility of hCMEC/D3 cells to EV strains was assessed in duplicate at 24 h post-infection (p.i.) by measurement of the production of viral RNA and infectious virus (Fig. S1). The virus yield exhibited a positive correlation (Spearman’s ρ 77 %, P<0.001) with viral RNA production (Fig. 1a). The virus strains selected amongst the EV-B types displayed different replication patterns defined by two arbitrarily selected thresholds of 0.00 log_{10} infectious particles per cell and 3.00 log_{10} genome copies per cell. The highest infectivity rates (from −0.55 to 2.86 log_{10} infectious particles per cell and 2.12 to 5.53 log_{10} genome copies per cell) were determined for the epidemiologically infrequent types echovirus (EV-B) E-1 and EV-B69 and the epidemic types E-6, E-11, E-12, E-13 and E-30. The strains selected amongst CV-B and EV-A types displayed the lowest infectivity rates (from −2.89 to 0.01 and −2.38 to −0.41 log_{10} infectious particles per cell and −0.44 to 3.53 and 2.01 to 4.51 log_{10} genome copies per cell, respectively).

Kinetics of viral RNA production performed in triplicate for EV-A71, E-6, E-30 and E-12 strains showed the highest rates of virus replication between 2 and 6 h p.i. (P=0.001; Fig. 1b). Different peaks of viral RNA production were reached at 24 h p.i. for the viruses tested (mean ± SD in log_{10} copies per cell): EV-A71 (3.29 ± 0.58), E-30 (4.15 ± 0.30), E-6 (5.30 ± 0.25) and E-12 (5.56 ± 0.21). These RNA levels were consistent with those obtained in Fig. 1(a): 2.46 ± 0.63, 3.85 ± 0.26, 5.02 ± 0.15 and 5.46 ± 0.15, respectively.

We determined the number of infected cells at 6 h p.i. (before extensive release of virus progeny) to investigate whether the variations in susceptibility of hCMEC/D3 cells
were related to differences in the infection efficiencies of EV strains. The infected cells were numbered in triplicate by computer-assisted image processing of low-magnification epifluorescence pictures. The highest proportion of infected cells (>30%) was determined for E-19, EV-B69 and E-1, and intermediate proportions of 10–30% were obtained with E-12 and E-16 (Fig. 1c). Less than 10% of infected cells were counted for virus strains of various types (E-30, E-3, E-7, CV-B6, E-4, E-14, E-18, EV-A71, E-9, E-32, CV-B3 and E-25). About 20 infected cells cm⁻² were counted for E-27 and E-11, and only 3–5 infected cells cm⁻² for EV-B70, EV-B77, CV-A9, E-15, E-24 and E-26 (data not shown). Two E-6 and E-13 strains exhibited different infection efficiencies (E-6/CF2660-01, >30%; E-6/CF158061-11 and E-13/CF1274-00, 10–30%; E-13/CF1925-01, <10%). The overall data suggested variations in the susceptibility of the hCMEC/D3 cells to infection by different EV types and subtypes.

**Cell mortality during virus infection and multidimensional analysis of EV infectivity**

The mortality rates of infected hCMEC/D3 cells were determined in quadruplicate at 24 h p.i. for a subset of 15 EV strains representative of different susceptibility patterns determined above in hCMEC/D3 cells (Fig. 1d). The mortality threshold was defined by the highest value of the SD calculated with mock-infected cells (i.e. 10%). High cell mortality rates >50% were found with E-1 and EV-B69, and cell death resulted from both necrosis and apoptosis.

Intermediate cell mortality rates between 40 and 50% were estimated for E-12, E-6/CF2660-01 and E-30/CF282-97. Other virus strains of the two latter types caused lower cell mortality (30–40%). Two E-13 strains were related to different cell mortality rates (E-13/CF1274-00, 38.3%; E-13/CF1925-01, <30%). Mortality of cells infected with EV-A71 (11%) was similar to that of mock-infected cells.

We used principal component analysis (PCA) to visualize on a map the ordination of the 15 EV strains according to the proportion of infected cells, production of viral genomes, yield of infectious particle production and cell mortality rates (Fig. S2). The proportion of infected cells and cell mortality exhibited a positive correlation, hereafter designated cell sensitivity (x-axis). The production of viral genomes and that of infectious virus were positively correlated, and designated viral replication (y-axis). As cell sensitivity and viral replication were orthogonal, they were not correlated with each other. PCA confirmed that the hCMEC/D3 cell line displayed large differences in sensitivity to EV types and strains within the same type (Fig. 2). We selected viral strains representative of different PCA patterns for further investigations with endothelial barriers (Table S2).

**Variations in permeability and structural integrity of endothelial barriers amongst EV types**

We prepared endothelial barriers *in vitro* with the hCMEC/D3 cells (Fig. S3) and quantified infection with five EV
types so that infection and paracellular permeability were assessed in the same samples. The structural features of mock-infected endothelial monolayers and their restrictive permeability were analysed using transmission electron microscopy (TEM) and clearance of a non-permeable fluorescent compound (Figs S3 and S4). Endothelial barriers infected with E-6, E-11, E-12 and E-30 strains exhibited little change in paracellular permeability at 24 h p.i., but permeability progressively increased afterwards (Fig. 3a–d). A release of viral genomes (>6 log_{10} copies) was detected at 6 h p.i. at both barrier sides, but in the abluminal compartment, viruses were detectable below the titration threshold for E-12 and E-30. The release of infectious progeny reached the highest levels at 24–48 h p.i. Scanning electron microscopy (SEM) allowed the identification of three main cytological alterations (Fig. 4). Compared with mock-infected controls, which had the appearance of joined cobblestones (Fig. 4a–c), the infected endothelial barriers exhibited cells with structural features suggestive of necrosis (damage plasma membrane) and apoptosis (preserved and budded plasma membrane), indicated by red and green arrowheads, respectively, in

![Graphs showing EV genome copies/infectious particles (log) per compartment and Permeability coefficient (10^-3 cm min^-1) over time.](image)

**Fig. 3.** Disruption of the microvascular endothelial barrier during EV infection. Endothelial barriers of hCMEC/D3 cells produced on Transwell membranes were infected with (a) E-6/CF2660-01, (b) E-11/CF228046-07, (c) E-12/CF1157-91, (d) E-30/CF2575-00, (e) EV-A71/CF166105-10 and (f) EV-A71/CF160019-10. At the indicated time points, the culture media in the luminal and abluminal compartments were collected and stored separately. Paracellular permeability was measured. Data represent the mean ± SD of three experiments. The numbers of EV genome copies and infectious virus particles are indicated by white and dashed bars, respectively, for the luminal compartment, and by dark grey and shaded bars, respectively, for the abluminal compartment. Permeability coefficients for mock-infected and infected barriers are indicated by green and red lines, respectively.
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(a) (b) (c)

E-6/CF2660-01

d) (e) (f)

g) (h) (i)

E-12/CF1157-91

(j) (k) (l)

E-30/CF2575-00

(m) (n) (o)

EV-A71/CF166105-10

Mock
Fig. 4. Disruption of endothelial barriers during EV infection. The hCMEC/D3 barriers were analysed using SEM at 24 h p.i. Representative fields of duplicate experiments are shown. (a–c) Mock-infected monolayers, and (d–l) barriers infected with E-6/CF2660-01, (g–i) E-12/CF1157-91, (j–l) E-30/CF2575-00 and (m–o) EV-A71/CF166105-10. White arrowheads, breach of the endothelial barriers; red and green arrowheads, cells with a necrotic and an apoptotic shape, respectively; blue arrowheads, round cells with no sign of altered plasma membrane.

Fig. 4(d–l). Rounded cells without apparent altered plasma membrane were suggestive of early steps of cell death (indicated by blue arrowheads). On the basis of these analyses, we found evidence of large amounts of altered cells, which caused breaches within endothelial barriers, as indicated by the visualization of pores of the microporous membrane (indicated by white arrowheads).

The paracellular permeability of endothelial barriers infected with the EV-A71 strains (genogroups C1 and C2) was maintained at levels similar to those of mock-infected barriers until 96 h p.i. (Fig. 3e, f). The abluminal release of EV-A71 genomes and infectious virus was the highest at 24 h p.i., and remained constant at slightly lower levels up to 96 h p.i. The infectious progeny were below the titration threshold at the abluminal side after 24 h p.i., but were consistently determined at low levels at the luminal side. SEM observations showed few groups of infected cells and a limited number of small breaches (Fig. 4m–o).

Intracellular changes in endothelial barriers during EV infection

The endothelial barriers were analysed by TEM to visualize the intracellular features of infected hCMEC/D3 cells and to investigate variations between EV types. The altered cells of endothelial barriers infected with the E-6/CF2660-01 strain displayed features indicative of virus infection, which were similar to those caused by E-30 and E-12 (data not shown). At 24 h p.i., the impaired cells displayed shrunken nuclei relocated near the cell membrane and contained myriad of virus-induced vesicle-like membranous structures, 200 nm in diameter (Fig. 5a, b). These structures had either single or double membranes and were organized in extensive intracellular arrangements (Fig. 5c). Some infected cells showed evidence of tubular structures with positive membrane invagination that enclosed cytoplasmic components (Fig. 5d). Clusters of electron-dense granules between membranous structures were suggestive of viral particles (Fig. 5e). Mitochondria were grouped near the membranous structures, which contrasted with mock-infected cells in which mitochondria formed an extensive network (Fig. S4). Large single-membrane vesicles (600–1000 nm in diameter) contained electron-dense cytoplasmic material and multilamellar structures resembling autophagic vacuoles (Fig. 5c).

In contrast to the features described above, the impaired cells of endothelial barriers infected with EV-A71/CF166105-10 displayed a number of structural variations. As shown in Fig. 6(a–d), the EV-A71-infected cells maintained an elongated shape and contained nuclei similar in shape to those seen in control barriers. Remodelling of intracellular components included vesicle-like structures and mitochondria clustered near the nucleus, whereas EV-A71-induced membranous structures had a uniform round shape with a diameter of 500 nm and a multilamellar structure (Fig. 6e–i).

Intracellular injury patterns common to EV types

The early virus-induced intracellular injuries at 6 h p.i. were further analysed by confocal microscopy, and viral replication was detected by staining VP1 protein. The infected cells displayed a major reduction in staining of polymerized actin in comparison with controls (Fig. 7a–r versus s–u). This indicated effective cytoskeleton impairment

Fig. 5. Ultrastructural features at 24 h p.i. of hCMEC/D3 barriers infected with an E-6 strain. (a, b) The infected hCMEC/D3 barriers were observed at low magnification with TEM. (c) Virus-induced reorganization of cytoplasmic elements. (d) Features of the vesicular structures. (e) Dense electron punctuation suggestive of virus aggregates. Bar, 4 μm (a, b), 500 nm (c–e). Representative fields of duplicate experiments are shown; mock-infected cells are shown in Fig. S4. N, nucleus; M, mitochondria; MC, membranous replication complex; A, autolysosome/amphisome; V, virus aggregates.
Early after the initiation of viral protein synthesis and would explain the subsequent cell rounding.

Early virus-induced changes in the mitochondrion network were analysed with a fluorescent probe that accumulated in active mitochondria. Mitochondria were stained in all virus-infected cells (Fig. 8a–o); however, in contrast to mock-infected controls, they were clustered in a perinuclear area (Fig. 8p–r). Rearrangement of the mitochondrion network was marked in cells exhibiting prominent staining of VP1 protein at 6 h p.i. (indicated by white arrowheads). Cells with reduced VP1 staining exhibited no or minor changes in the mitochondrion network (indicated by yellow arrowheads). Mitochondrion clustering was dependent on viral replication intensity, but was not directly related to cell rounding, as cells that were not yet round also displayed clustered mitochondria.

**DISCUSSION**

Human EV infections are associated with meningitis, encephalitis and encephalomyelitis, but our current knowledge about CNS invasion by enteric viruses is still scant. The BBB may represent a common entry pathway for EVs during viraemia, which precedes disease onset. In this study, we used the hCMEC/D3 human cerebral microvascular endothelial cell line as a model system for investigating EV entry routes into the CNS through the human BBB. We showed that the hCMEC/D3 cells were permissive to infection by a large array of EVs, and found major differences between types and genogroups. Most EV strains occupied a central position in the susceptibility spectrum of hCMEC/D3 cells, notably the E-6, E-13 and E-30 strains, and intratypic variations may be related to individual genetic differences amongst genogroups and sub-genogroups. A wide range of cellular receptors has been observed in human EVs (reviewed by Merilahti et al., 2012). Although we did not examine the binding processes of EVs to the hCMEC/D3 cell surface, there is a large body of earlier experimental evidence to suggest that the intertypic variations in hCMEC/D3 susceptibility to EVs can be attributed to their propensity for using a wide range of receptors and internalization processes (Coyne et al., 2007; Bozym et al., 2010; Ylipaasto et al., 2010). For example, E-1 stands apart within the susceptibility spectrum of hCMEC/D3 cells to EV infection – a pattern that may be related to the fact that it is the only type known to bind integrin α2β1 (Bergelson et al., 1993). A number of EV types examined in our study (E-6, E-11, E-12, E-13 and E-30) bind the same cellular receptor CD55 (Bergelson et al., 1994). However, the virus strains of these types did not cluster in the same area of the susceptibility spectrum of hCMEC/D3 cells. This suggests that additional factors other than canonical receptors should be considered and that genogroup features may be involved.

Our investigation provides evidence of two major clusters amongst EV types. A first cluster consists of the EVs that exhibit a highly cytolytic phenotype, produce infectious progeny and induce extensive disruption of the endothelial barrier. Early during cellular infection by these viruses, the amount of virus genomes released in the abluminal compartment was >10 000 times higher than that of infectious progeny. We assumed that paracellular transport of viral RNA and defective virus particles were not involved because barrier permeability to the fluorescent reporter was not yet compromised at this time – a hypothesis that is also supported by SEM observations. At 24 h p.i., the difference between the release of viral genomes and virus particles was substantially reduced at the abluminal side as a result of the destruction of the endothelial barrier caused by infected dying cells. In contrast, the release of genomic material was relatively constant over time at the luminal sides. Accordingly, massive amounts of viral genomes appeared to drain off the cells through their basolateral membrane early during infection by an as-yet unknown process.
Fig. 7. The actin cytoskeleton network is disrupted in EV-infected hCMEC/D3 cells. The hCMEC/D3 cells were analysed at 6 h p.i. during replication of strains (a–c) E-12/CF1157-91, (d–i) E-6/CF2660-01, (j–l) E-30/CF2575-00, (m–o) E-11/CF228046-07 and (p–r) EV-A71/CF166105-10; (s–u) mock-infected cells. Actin network, red, VP1 capsid protein, green; nuclei, blue. Bar, 10 μm. White arrowheads indicate intermediate disruption of the actin cytoskeleton.
Fig. 8. Perinuclear relocation of active mitochondria in EV-infected hCMEC/D3 cells. The hCMEC/D3 cells were analysed at 6 h p.i. during replication of strains (a–c) E-12/CF1157-91, (d–f) E-6/CF2660-01, (g–i) E-30/CF2575-00, (j–l) E-6/CF2660-01, (m–o) E-30/CF2575-00, and (p–r) mock.
The second cluster comprises CV-B and EV-A71 types, which produced no impairment of the in vitro model of endothelial barrier. A key observation, in sharp contrast to the above data, is that hCMEC/D3 cells are moderately permissive to EV-A71 infection. This pattern resulted from a high replication rate of the viral genome, but a remarkably poor production of infectious viruses. Both virus and viral RNA were released from the luminal and basolateral sides of the endothelial barrier, but at disproportionately different rates as the infectious virus was consistently detected at minute amounts. This process was maintained for at least 4 days and did not induce a breakdown of the barrier nor change the paracellular permeability as measured with the Lucifer Yellow (LY) surrogate marker. This non-disruptive pattern occurred even when the barriers were inoculated at m.o.i. ~100 TCID₅₀ per cell (data not shown), which suggests that it was not dependent on the initial infection conditions but was more probably related to post-entry factors. A non-disruptive and long-term replication pattern was also shown for CV-B3 and CV-B5 (data not shown). A persistent replication was reported earlier for CV-B3 and CV-B4 in human dermal microvascular endothelial cells (Zanone et al., 2003). A member of the genus Flavivirus, West Nile virus (WNV), can cross in vitro BBB models by infection of endothelial cells (Verma et al., 2009). The brain endothelium crossing and infection by WNV is not related to direct disturbance of the endothelial barrier integrity in vitro, as observed in our study for EV-A71 and CV-B. The loss of BBB integrity associated with the WNV may be related to upregulation of cell adhesion molecules (e.g. vascular cell adhesion molecule-1, E-selectin) in infected endothelial cells, thus promoting transendothelial migration of leukocytes (Verma et al., 2009, 2010).

The infected cells showed typical ultrastructural features of a picornavirus infection. We found evidence for both apoptosis and necrosis amongst infected cells regardless of the EV type, in agreement with data indicating a competition between cell death pathways and picornavirus replication (Agol & Gmyl, 2010). We also observed disruption of the actin cytoskeleton network and that of intercellular junctions as evidenced by the rounding of infected cells. The actin cytoskeleton has an important role in the maintenance of stable inter-endothelial junctions and prevents paracellular transport to the brain (Spindler & Hsu, 2012; Stamatovic et al., 2012). Remodelling of intracellular membranes was the third hallmark of a picornavirus infection seen in infected hCMEC/D3 cells, but discrete variations occurred between echoviruses (E-6, E-12 and E-30) and EV-A71. In the echovirus infections, we found evidence of single- and double-membrane vesicles organized in compact arrangements near the nucleus and of structures that displayed positive membrane invagination. Both features were reported previously in Vero and HeLa cells infected by CV-B3 and PV-1, respectively (Limpens et al., 2011; Belov et al., 2012). The vesicular structure in the EV-A71-infected hCMEC/D3 cells was characterized by less condensed vesicles and an increased proportion of multilamellar and large vesicles. Finally, our analyses with TEM and fluorescence microscopy showed clustering of the mitochondrial network – a previously unobserved feature of EV infections that occurred early during the virus infection and whose origin is still unclear. It may be related to virus-induced disruption of microtubules, as suggested for cells infected with Hepatitis B virus (family Hepadnaviridae) (Kim et al., 2007). A similar feature was also reported for African swine fever virus (family Asfarviridae), another DNA virus, and was related to coupling between viral translation and ATP synthesis (Rojo et al., 1998).

The most frequent EV infections of the CNS cause meningitis as a result of virus replication in the cells of the leptomeninges (the innermost layers of the meninges) (Rotbart, 1995). These infections are usually self-limited because the meninges are directly accessible to immunological surveillance and subject to rapid immune responses (Engelhardt & Coisne, 2011). The varying amounts of viruses in the CSF of patients with EV meningitis within a few hours after the onset of symptoms (Volle et al., 2014) may reflect virus unloading from these infected sites. The meningeal blood vessels, which form the barrier between blood and CSF, are only made of non-fenestrated endothelial cells; this contrasts with the BBB, which includes other cell types. Accordingly, our endothelium model is consistent with the blood–CSF barrier. The infection of endothelial cells reported in this study for a large array of EV types may occur during the earliest stages of viraemia, which develops following EV replication in peripheral tissues. The local EV replication may contribute to infection of leptomeninges and the development of a neuro-inflammatory disease. Of note, regional blood flow reduction and cerebral vasculitis can be observed in children with E-30 aseptic meningitis (Nishikawa et al., 2000). Care must be taken in making generalized conclusions of pathophysiology based on in vitro model systems and the transcellular passage for neural spread of EVs requires close examination in an appropriate in vivo model.

**METHODS**

**Cell lines and viruses.** hCMEC/D3 human cerebral microvascular endothelial cells were grown in EBM-2 basal medium (Lonza) supplemented with 5% FBS, 1% penicillin (10,000 U), 1% streptomycin (10 mg ml⁻¹); GE Healthcare Life Science, 1% streptomycin (10 mg ml⁻¹); GE Healthcare Life Science, 1%...
chemically defined lipid concentrate (Invitrogen), 10 mM HEPES, 
1.4 μM hydrocortisone (Sigma-Aldrich), 1.5 μg ascorbic acid ml⁻¹ 
(Sigma-Aldrich) and 200 ng basic fibroblast growth factor ml⁻¹ 
(Sigma-Aldrich). The cells were seeded for all experiments on rat 
collagen I-coated culture surfaces (R&D Systems). The rhabdomyo-
sarcoma (RD) cells were grown in RPMI 1640 medium (Lonza) with 
1% penicillin/streptomycin and 4% FBS. The buccal epithelial 
carcinoma (KB) cells were grown in Dulbecco’s modified Eagle’s 
medium basal medium (GE Healthcare Life Science) with 1% 
penicillin/streptomycin and 6% FBS. All cell cultures were main-
tained at 37 °C in a humidified atmosphere containing 5% CO₂.

A sample of 88 EV strains, comprising 23 reference strains and 65 
clinical isolates, recovered from patient specimens (CSF, stool or 
throat) was used in the study (Table S1). Virus stocks were prepared 
with KB cells (coxsackievirus B) and RD cells (other EVs) and stored 
at −20 °C. Titration of viral suspensions was done using our end-
dpoint dilution assay (Bailly et al., 1991). The cell cultures were 
incubated at 37 °C for 1 h before fixation, with complete EBM-2 
solution. After three PBS washes, nuclear DNA was counterstained 
with Hoechst compound, apoptotic when only stained with the Annexin V-
conjugated antibody or necrotic when they were stained with both 
Annexin V and ethidium homodimer III. Cell fragments were 
detected through ethidium homodimer III staining alone.

**Extraction of nucleic acids and EV quantitative real-time (qRT)-
PCR.** Nucleic acids were extracted from 200 μl supernatant or the 
whole cells and supernatant using a NucliSens EasyMAG extractor 
(bioMérieux) and were eluted with 25 μl elution buffer provided by 
the manufacturer. A previously described competitive internal control 
was added during the extraction step and amplified in our in-house 
qRT-PCR assay (Volle et al., 2012).

**Viability of infected hCMEC/D3 cells.** Cells were infected 
separately by 15 EV strains. After two washes at 24 h p.i., the cells 
were detached, centrifuged for 10 min at 1000 g, stained with an 
Apoptotic/Necrotic/Healthy Cells Detection kit (Promokine) and 
analysed by flow cytometry (LSR II; BD Bioscience). Cells were 
considered as being viable when only stained with the Hoechst 
compound, apoptotic when only stained with the Annexin V-
conjugated antibody or necrotic when they were stained with both 
Annexin V and ethidium homodimer III. Cell fragments were 
detected through ethidium homodimer III staining alone.

**Fluorescent microscopy.** hCMEC/D3 cells were grown in chamber 
slides, infected for 6 h by different EV strains and fixed with 4% 
paraformaldehyde for 10 min. For mitochondria staining, the cells 
were incubated at 37 °C for 1 h before fixation, with complete EBM-2 
medium containing 50 nM MitoTracker Mitochondrion-Selective 
Probe M7510 (Invitrogen) in a humidified atmosphere of 5% CO₂. 
Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, 
saturated for 10 min with 5% BSA in PBS and incubated overnight at 
4 °C with mouse primary mAbs against the EV capsid protein VP1 
(Diagnostic Hybrid). After three PBS washes, incubation was pursued 
for 1 h at 37 °C in a solution of anti-mouse secondary antibodies 
conjugated to DyLight 488 (anti-corps-ennigle.fr). In the tests for 
which active mitochondria staining was not required, red phalloidin 
used for actin staining was included in the secondary antibody 
solution. After three PBS washes, nuclear DNA was counterstained 
with a Hoechst solution (Promokine). The slides were mounted with 
coverslips and observed with an epifluorescence microscope (BX41; 
Olympus) or scanning confocal microscope (LSM 510; Carl Zeiss 
MicroImaging). Automated image analysis (FIJI software) was used to 
calculate the number of infected cells.

**EV crossing through an in vitro model of the brain micro-
vascular endothelial barrier.** To obtain microvascular endothelial 
barriers, hCMEC/D3 cells were cultured on a permeable membrane 
(0.4 μm pore) placed in the upper chamber of a Transwell device (12-
well plate; Corning). The upper chamber was seeded with 40,000 cells 
per cm² and incubated for 5–7 days to obtain a tight confluent cell 
monolayer. In this in vitro model, the cells were polarized and 
displayed a luminal side and an abluminal side (Wekslar et al., 2005). 
The luminal sides of non-permeable barriers were exposed separately 
to various EV strains, the infected barriers were incubated for the 
indicated times and permeability was determined at each time point 
post-infection (see below). The yield of infectious particles and the 
total amount of viral genome released through the abluminal and 
luminal sides were determined as described above.

**LY permeability assay.** The paracellular seal of the endothelial 
barrier was determined in triplicate by testing the permeability to the 
LY marker (Sigma). The cell monolayers were washed twice with 
collecting buffer consisting of Hank’s balanced salt solution (GE 
Healthcare Life Science) supplemented with 1% HEPES (GE 
Healthcare Life Science) and 1% sodium pyruvate (GE Healthcare 
Life Science). The LY marker (50 μM; 400 μl) was added to the upper 
chamber. Cells were incubated at 37 °C (5% CO₂ and 100% 
humidity) in three successive collecting wells, each containing 1.6 ml 
collecting buffer, for 10, 15 and 20 min, respectively. The LY 
centrations in the collecting buffer of each well and the stock 
LY solution were determined by fluorometry. Parallel negative control 
tests were performed with cell-free collagen-coated culture mem-
branes. Samples were analysed in black 96-well microtitre plates using 
a Fluorescan Ascent FL fluorometer (Thermo Electron) at 485 and 
538 nm wavelengths for excitation and emission, respectively.

**SEM and TEM.** The endothelial barriers were washed with 0.2 M 
sodium cacodylate buffer (NCD; pH 7.4) and fixed overnight at 4 °C 
in 1.6% glutaraldehyde-NCB. The cells were fixed for 1 h with 1% 
OsO₄ in NCB. For SEM preparation, cells were dehydrated in graded 
ethanol, followed by critical-point drying with 100% ethanol and 
hexamethyldisilasane (1:1) for 10 min, sputter-coated with gold 
(JFC-1300; JEOL), and observed at 5 kV with a JEOL 6060-LV 
microscope. For TEM preparation, cells were dehydrated in graded 
ethanol, infiltrated sequentially with three mixtures of ethanol/EPON 
resin (2:1, 1:1 and 1:2) for 1 h each, embedded in EPON resin 
overnight at room temperature and cured for 2 days in a 60 °C oven. 
Thin sections (70 nm; UC6 ultramicrotome; Leica) were stained with 
uranyl acetate and lead citrate, and observed at 80 kV with a Hitachi 
H-7650 microscope. All chemical products were provided by Delta 
Microscopies.

**Statistical analysis.** Statistical analyses were performed with Stata 
(version 12; StataCorp). Tests were two-sided, with a type I error set 
at α=0.05. Quantitative data were expressed as means (and associated 
SD), Gaussian distribution verified by the Shapiro–Wilk test. 
Correlated data were analysed by mixed models to study the 
evolution of parameters taking into account between- and within-
strain variability (random effects such as intercept and slope). 
These analyses were completed by ANOVA for repeated measures followed by a post hoc Tukey–Kramer test. PCA was undertaken to explore the 
relation between several quantitative parameters. Correlation coeffi-
cients (Pearson or Spearman when appropriate) were calculated to 
quantify these relations.

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